INFECTION AND IMMUNITY, Jan. 1997, p. 171–178 Vol. 65, No. 1
0019-9567/97/$04.00+0

Vaccine Efficacy of *Salmonella* Strains Expressing Glycoprotein 63 with Different Promoters

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Received 1 July 1996/Returned for modification 30 July 1996/Accepted 10 October 1996

The development of *Salmonella* vaccine vectors has been hindered by both the requirement for multiple doses to induce immune responses and a lack of plasmid stability. Direct comparisons of different promoter systems with the same antigen are necessary to address these important issues. We have previously described an AroA$^-$ AroD$^-$ deletion mutant of *Salmonella typhimurium* (GID101) which expresses the gene encoding the *Leishmania major* promastigote surface glycoprotein gp63 (GID101). While this construct provided significant protection against *L. major* challenge to highly susceptible BALB/c mice, this required at least two oral doses. We report here the use of two different inducible promoters, the *nirB* and *osmC* promoters, to improve vaccine efficacy. These constructs (termed GID105 and GID106, respectively) expressed gp63 in vitro under inducible conditions and colonized BALB/c mice after oral administration. GID105 demonstrated greater plasmid stability in vitro and in vivo than did either GID106 or GID101, which expresses gp63 constitutively. Spleen and lymph node cells from mice immunized with a single oral dose of GID105 proliferated in vitro in response to *L. major* and secreted gamma interferon, whereas cells from mice given the other constructs did not. Mice immunized with a single oral dose of GID105 or GID106 developed significantly smaller lesions upon challenge with *L. major*, whereas mice administered GID101 did not. Mice administered GID105 also showed considerable resistance to *Leishmania donovani* infection. These data provide a direct comparison of promoter systems and demonstrate that the use of inducible promoters such as the *nirB* promoter allows a considerable improvement over the previous vaccine construct in terms of protection against infection.

Despite recent advances in understanding the nature of protective immune responses against leishmaniasis, there remains no prophylactic treatment against any form of the disease, with the exception of deliberate infection with live pathogenic organisms. We have previously reported the construction of the *Salmonella typhimurium* AroA$^-$ AroD$^-$ strain GID101, which constitutively expresses the major surface glycoprotein, gp63, of *Leishmania major* (39). After two oral doses of this construct, significant protection against *L. major* challenge infection was conferred on highly susceptible BALB/c mice.

One potential drawback in using vaccines with live bacteria such as *S. typhimurium* for antigen delivery is the instability of foreign antigen expression in vivo. Unregulated expression of high levels of a foreign protein in bacteria from multiple-copy plasmids often results in rapid loss of the plasmid or expressed gene from the bacteria. This problem can be controlled in vitro by repeated passage in broth (7) or by the use of plasmids carrying essential genes (27) or on-off promoters (37). Plasmid stability can also be improved by using inducible promoter systems such as the p_L, *trp*, *lac*, and hybrid *trp-lac* systems (1, 32), which rely on either a temperature shift or the addition of a chemical to induce their activity. These systems are clearly impractical when bacteria are growing in host tissues during the self-limiting growth following vaccination. Introduction of genes into the bacterial chromosome by homologous recombination (14, 35) can achieve a high degree of stability, but this approach is limited by low protein expression due to a single gene copy.

The most desirable method of stabilizing antigen expression may be to use an in vivo-inducible promoter. Ideally, it would be a promoter which is activated efficiently only when bacteria enter the host or even when they interact with immune cells. The bacterial *nirB* (15) and *osmC* (10) promoters are known to be sensitive to changes in the extracellular environment and thus may be useful in the building of stable vaccine constructs.

The *nirB* promoter has been isolated from *Escherichia coli*, where it directs expression of an operon which includes the nitrate reductase gene (15). It is regulated by nitrate and by changes in the oxygen tension of the environment, becoming active under anaerobic conditions (6). The *osmC* promoter and gene, induced by an increase in osmotic pressure, have been cloned from *E. coli*. The promoter is composed of two overlapping promoters, $osmCp_1$ and $osmCp_2$, located within a 137-bp DNA fragment (10).

Several previous reports compared the use of promoters of different strengths (4, 9, 12, 16, 18, 19, 28, 38) to express foreign antigens and for chromosomal expression (13) in salmonellae. However, the relative efficacies of promoters and the mechanisms underlying immunogenicity remain unclear.

Two or more doses of the previously reported construct, GID101, are required to have any prophylactic effect on *L. major* infection. Thus, we have used the *nirB* and *osmC* promoters in order to construct strains of *S. typhimurium* which express gp63 to try to improve vaccine efficacy. We report a direct comparison of the growth, plasmid stabilities, and immunogenicities of the strains in addition to their protective capacities against experimental leishmaniasis.

MATERIALS AND METHODS

Mice. Inbred BALB/c mice were obtained from Harlan Olac (Bicester, United Kingdom). Female mice 6 to 8 weeks old were used throughout.

Parasites and antigens. The *L. major* isolate MRHO/SU/59/P, also known as LV39, was used throughout. The maintenance, cultivation, and isolation of *L. major* promastigotes have been described in detail elsewhere (23). Soluble anti-

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TABLE 1. *Salmonella* strains used in this study

Strain	Relevant properties	Source and/or reference
LB5010	Wild-type *S. typhimurium* strain	Genetic Stock Centre, University of Calgary, Canada; provided by S. Chatfield (Medeva, London, United Kingdom) (2)
BRD509	SL1344 AroA$^-$ AroD$^-$	G. Dougan, Biochemistry Department, Imperial College of Science, Technology and Medicine, London, United Kingdom (34)
GID101	pkk-Imm63-67 in BRD509	Department of Immunology, Glasgow University, Glasgow, United Kingdom (39)
GID105	pnirB/gp63 in BRD509	This study
GID106	posmC/gp63 in BRD509	This study

gen was prepared from parasites by five cycles of freezing and thawing (2×10^8/ml in phosphate-buffered saline [PBS]) followed by centrifugation at 8,000 × *g* for 10 min at 4°C. The supernatant was filtered (0.45-μm-pore-size filter) and stored at −70°C.

Construction of the pnirB expression vector. The expression plasmid pTET nir15 was obtained from S. Chatfield (5). This plasmid (3.7 kb) expresses the tetanus toxin fragment C gene under control of the *nirB* promoter. To make it more suitable for use as a vector for expression of foreign genes, the plasmid was reconstructed by removing the fragment C gene and introducing unique restriction enzyme sites downstream of the promoter region. The plasmid was cut with *Cla*I and *Bgl*II, and the 2.8-kb fragment was purified and ligated with a 32-bp synthesized DNA linker which contained a Shine-Dalgarno (SD) sequence (AG GAG); an *Nde*I site, including an ATG start codon, 9 nucleotides downstream of the SD sequence; and *Bgl*II and *Cla*I sites at the 5′ and 3′ termini, respectively. The ligated product was transduced into *E. coli* MM294, and ampicillin-resistant clones were further identified by restriction enzyme mapping. The clone that had the correct insertion was termed pnirB. The DNA linkers for the reconstruction were 5′-GATCTTAATCATCCACAG-GAGACTTTCATATGAT-3′ and 3′-A ATTAGTAGGTGTCCTCTGAAACTATACT-AGC-5′ (synthesized by Genosys Biotechnologies, Inc., Cambridge, United Kingdom).

Cloning of the *S. typhimurium osmC* promoter region (*osmCp*$_1$ and *osmCp*$_2$) and construction of posmC. The *osmC* promoter region (*osmCp*$_1$ and *osmCp*$_2$) was amplified from *S. typhimurium* LB5010 (*galE* r$^+$ m$^+$) by PCR. The sense and antisense PCR primers were designed according to the published *osmC* promoter sequence in *E. coli* (10). Primers used for cloning and sequencing of the *osmC* promoter from *Salmonella* were as follows: sense, 5′-GGAATTCAATTT AAGTGCAGAAGTA; antisense, 3′-GGATCCCCAGATCTTATCGATTGTC AGAGGATCCGATCTCCT. PCR was carried out as follows. Five microliters of an overnight culture of LB5010 was used as a source of template DNA and amplified in 10 mM Tris–50 mM KCl–1.5 mM $MgCl_2$–40 nM deoxynucleoside triphosphates–0.2 μM each primer–2 U of *Taq* DNA polymerase. The PCR program was 95°C for 1.5 min, 47°C for 1.5 min, and 73°C for 1.5 min for 30 cycles. The product of the amplification of the *osmC* promoter region was a DNA fragment of approximately 100 bp which was further identified by DNA sequencing. The result showed that this fragment from *S. typhimurium* contained a complete *osmC* promoter region as in *E. coli*. It was composed of −35 and −10 hexanucleotide regions of *osmCp*$_1$ and *osmCp*$_2$, separated by 17 nucleotides, and a typical SD (AGGAG) located 7 nucleotides upstream of the initiation codon (ATG). The PCR fragment of the *osmC* promoter was purified by electroelution, digested with *Eco*RI and *Bgl*II, and then inserted into the plasmid pnirB at the *Eco*RI and *Bam*HI sites. The positive clones that contained the correct insert were named posmC.

Construction of recombinant *S. typhimurium*. *S. typhimurium* LB5010 (Table 1) (2), originally from the Genetic Stock Centre, University of Calgary, Calgary, Canada, was provided by S. Chatfield (Medeva, London, United Kingdom). The AroA$^-$ AroD$^-$ vaccine strain BRD509 (Table 1) has been described in detail elsewhere (34). We have previously described the vaccine strain GID101, which expressed gp63 constitutively by using the *tac* promoter (39). The high-transducing phage P22HT 105 int (8) was obtained from T. Foster, Trinity College, Dublin, Ireland. The plasmid Imm63-67 (3) was the kind gift of W. R. McMaster, University of British Columbia, Vancouver, Canada. Plasmid pTETnir15 (30) was the kind gift of S. Chatfield. Bacteria were routinely cultured on L agar or in L broth with or without 100 μg of ampicillin per ml. GID105 and GID106 were constructed with the gp63 gene inserted downstream of the *nirB* promoter in pnirB or the *osmC* promoter in posmC, respectively (Table 1). Plasmids were then transformed into LB5010 by the method of Lederberg and Cohen (21), selecting for resistance to ampicillin. Plasmids were transduced to BRD509 by P22 transduction (8).

Induction of gp63 expression in vitro. gp63 expression in GID105 containing the *nirB* promoter was carried out as previously described (30). Briefly, bacterial colonies were grown overnight at 37°C in L broth containing ampicillin and then diluted 1:100 with L broth containing ampicillin and 4 mg glucose per ml into a closed screw-cap container. Bacteria were incubated for 4 to 6 h at 37°C before screening. *S. typhimurium* GID106, containing the *osmC* promoter, was similarly grown overnight before dilution in L broth containing ampicillin and 0.3 M NaCl. Bacteria were incubated for a further 4 h at 37°C before screening.

Western blots. Bacterial lysates were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (20). Transfer to nitrocellulose was performed with a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.). Transferred proteins were detected by using a monoclonal anti-gp63 antibody (monoclonal antibody 235, kindly provided by W. R. McMaster, University of British Columbia). A rabbit anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad, Hemel Hempstead, Herts, United Kingdom) was then applied, and the blots were developed with horseradish peroxidase color-developing reagent (Bio-Rad). The level of gp63 synthesis in all strains was too low to be detected by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis of total cell lysates.

Plasmid stability in vitro. Initially, bacteria were grown in 5 ml of L broth without ampicillin for 24 h at 37°C. The next day, 1 μl of the culture was used to inoculate cultures for the following day, and the remaining L broth was serially diluted 10-fold in 1 ml of PBS without ampicillin, plated onto nonselective medium, and scored 24 h later. Plasmid segregation was determined by transferring 100 colonies from nonselective medium with or without ampicillin (100 μg/ml) and scoring the cultures 24 h later. This protocol was followed for seven consecutive days. Each cycle represents approximately 14 generations of growth for a total of about 98 generations.

Plasmid stability in vivo. The growth of the *Salmonella* constructs in vivo was monitored as previously described (29). Homogenates of spleen, liver, and mesenteric lymph nodes (MLN) were plated onto L-agar plates in the presence or absence of ampicillin to determine the number of colonies in these organs that had maintained the plasmid.

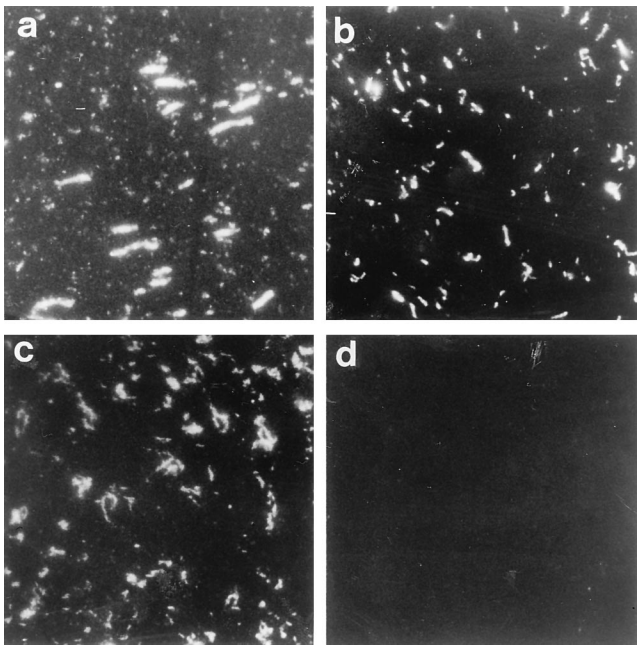

FIG. 1. Immunocytochemical analysis of gp63 expressed in transduced BRD509. Bacteria were cultured and induced as described in Materials and Methods. Cells were dispensed onto a glass slide, fixed with methanol, probed with anti-gp63 monoclonal antibody (1/500), and visualized with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (1/200). GID101 (a), GID105 (b), and GID106 (c) showed clear staining. No staining was detectable in BRD509 (d).

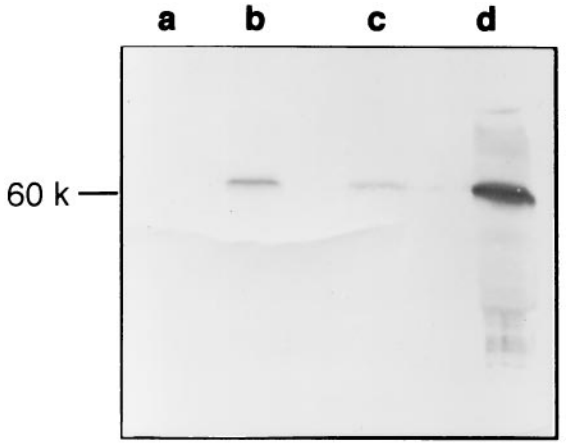

FIG. 2. Western blot analysis of gp63 expression in transduced BRD509. Bacteria were cultured and induced as described in Materials and Methods. They were lysed in sample buffer (10^6 cells/ml) and boiled for 5 min before being applied to an SDS–7.5% polyacrylamide gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane and probed with an anti-gp63 monoclonal antibody. Lanes: a, BRD509; b, GID106; c, GID105; d, GID101.

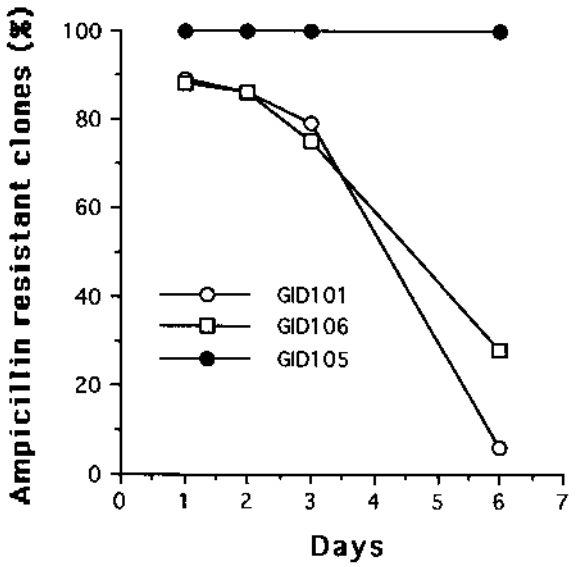

FIG. 3. Plasmid stability in vitro. The stability of plasmids was tested in vitro by growing bacteria from a single colony in ampicillin-free medium and passaging them each day into fresh medium. Cultures were inoculated onto ampicillin-free plates, and colonies were counted 24 h later. Plasmid segregation was determined by transferring 100 colonies from nonselective L-agar plate to plates with or without ampicillin, and colonies were counted 24 h later. Data are pooled from three experiments.

Vaccination of mice with *S. typhimurium*. Immediately before administration of *Salmonella* or PBS, mice were administered 100 μl of 5% Na_2CO_3 orally. Mice were then immunized orally with an overnight culture of 5×10^9 cells of GID105, GID106, GID101, or the control *Salmonella* strain, BRD509, in 0.2 ml of PBS. All oral inoculations were carried out with a stainless steel gavage needle without an anesthetic. The inoculum dose was checked by plating dilutions of each culture on L-agar plates with or without ampicillin. A group of mice was given a similar volume of PBS orally. One week after vaccination, two mice from each vaccinated group were sacrificed, and bacterial colonies in the MLN were counted to ensure that successful infection had occurred.

Proliferation assay. Spleen and MLN cells were harvested from mice 3 to 4 weeks after vaccination, and single-cell suspensions were obtained by gentle homogenization. The cells were washed twice with RPMI 1640 (Gibco, Paisley, United Kingdom) and resuspended at 4×10^6 cells/ml in culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM 2-mercaptoethanol). Cells (4×10^5 in 100 μl) were added to 96-well microtiter plates (Costar, Cambridge, Mass.) containing soluble leishmanaial antigen or live *L. major* promastigotes at various concentrations. The cultures were incubated for 3 to 5 days at 37°C in 5% CO_2 before being pulsed for 4 h with [^{3}H]thymidine (1 μCi per well) and harvested with an automatic cell harvester. Incorporated radioactivity was counted with a beta counter (Betaplate; LKB, Turku, Finland).

Assays for IL-2, IL-4, and IFN-γ. Cultures were set up in a manner identical to that for the proliferation assays. Cytokine levels in cell culture supernatants of cells stimulated with leishmanial antigens were measured by enzyme-linked immunosorbent assay (ELISA) with paired antibodies from PharMingen (Cambridge BioScience, Cambridge, United Kingdom). The cytokine content of each sample was established by comparison to a standard curve for the appropriate recombinant cytokine (gamma interferon [IFN-γ] was a kind gift from G. Adolf, Vienna, Austria; interleukin-2 [IL-2] and IL-4 were purchased from Genzyme, Kent, United Kingdom).

Measurement of delayed-type hypersensitivity (DTH) and antibody. Mice were injected in the right hind footpad with 50 μl of PBS containing 10^6 live promastigotes 3 to 4 weeks after vaccination. The footpad swelling was measured at regular intervals for up to 72 h with a spring-loaded dial calliper (Schnelltaster; Kroeplin, Surrey, United Kingdom). Leishmania-specific IgG was measured by an ELISA method involving 96-well plates coated with either recombinant gp63 or soluble *L. major* antigen, as previously described (40). Recombinant gp63 was kindly provided by W. R. McMaster, University of British Columbia.

Challenge with *L. major* or *Leishmania donovani*. Mice were challenged 3 weeks after vaccination by subcutaneous injection in the footpad with 10^6 stationary-phase *L. major* promastigotes in 50 μl of PBS. Lesion development was measured as an increase in footpad thickness, as described previously (22, 25). *L. donovani* amastigotes were isolated from the spleens of infected Syrian hamsters as previously described (17). Amastigotes were washed twice and resuspended at 5×10^7/ml before 200 μl (1×10^7 amastigotes) was injected intravenously into mice which had been vaccinated 3 weeks previously. Parasite numbers in the liver imprints of infected mice were counted and were expressed as *L. donovani* units, as previously described (33).

Statistical analysis. Statistical significance was analyzed with Student's *t* test. $P < 0.05$ is considered significant.

RESULTS

Expression of gp63 induced by transduced BRD509. gp63 was cloned into plasmids with the *nirB* or the *osmC* promoter as described in Materials and Methods. The *S. typhimurium* AroA$^-$ AroD$^-$ mutant (BRD509) was then transfected with either of these plasmids as described above. Expression of gp63 in these transfectants (GID105, pnirB/gp63; GID106, posmC/gp63) was induced and analyzed by immunocytochemical staining and Western blotting with an anti-gp63 monoclonal antibody. Expression of gp63 was evident by the bright fluorescent staining of the transfected bacteria compared with the control untransfected BRD509 (Fig. 1). Western blotting showed a single band at approximately 60 kDa in all of the transfectants, particularly GID101, which constitutively expresses a high level of gp63 (Fig. 2). Expression of gp63 in GID105 was not detected under noninducing conditions but was readily detectable after induction under anaerobic conditions. Expression of gp63 in GID106 was detectable under inducing and noninducing conditions, although there was considerable upregulation after induction (data not shown).

Plasmid stability. The stabilities of the plasmids in the three constructs were determined in vitro and in vivo. Figure 3 shows that the plasmid in GID105 was stably retained following extended culture without the selection pressure of ampicillin (for about 98 generations). In contrast, 96% of GID101 cells and 70% of GID106 cells lost their plasmids under the same culture conditions. Groups of mice were then orally administered 5×10^9 bacteria, and organs were harvested at various times to assess bacterial colonization and plasmid stability. The growth curves of GID105, GID106, and BRD509 in spleen, liver, and MLN are shown in Fig. 4. GID105 maintained the plasmid stably throughout the whole period of bacterial colonization, whereas GID106 experienced significant plasmid loss in vivo. There were also differences in the bacterial loads in the con-

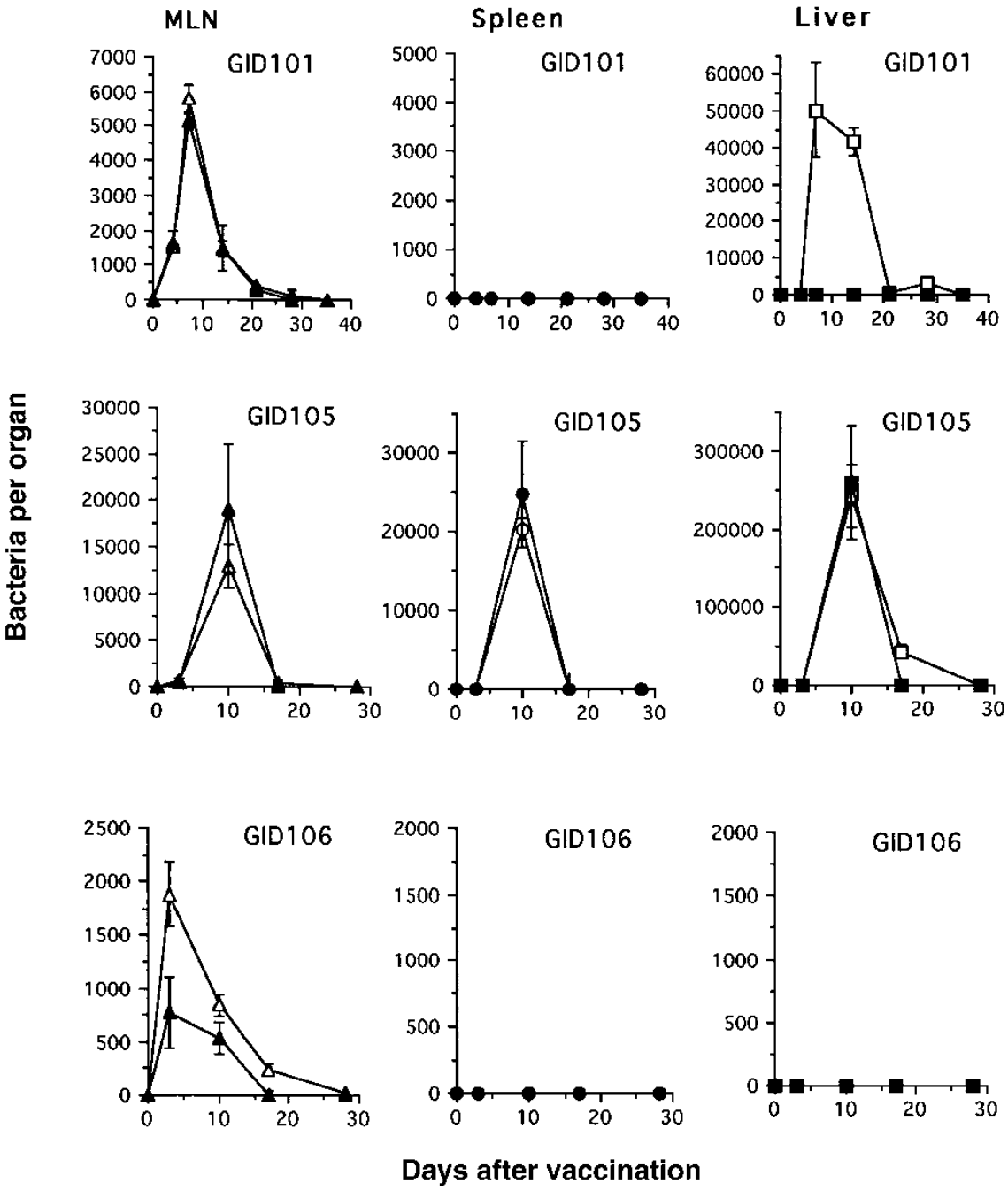

FIG. 4. Comparison of plasmid stabilities in vivo. Groups of BALB/c mice were vaccinated orally with 5×10^9 organisms/mouse. Spleens, livers, and MLN were removed at the times indicated, and CFU were assayed on plates with (closed symbols) or without (open symbols) ampicillin. Each point represents the geometric mean ± one standard deviation for three mice.

structs. Mice administered GID105 contained considerably more bacteria in spleen, liver, and MLN than those administered the control strain BRD509. Conversely, there was no colonization of spleen or liver by GID101 and GID106, although there was significant colonization of MLN by these two constructs. With all of the constructs, no bacteria were detected in any organs after day 28.

Antibody and DTH responses to gp63. BALB/c mice were immunized orally with 5×10^9 GID105, GID106, GID101, or BRD509 organisms or with PBS, and sera were analyzed for the presence of IgG to recombinant gp63 and soluble *L. major* antigens by an ELISA method. Sera from mice vaccinated 2 to 3 weeks previously with GID105, GID106, or GID101 contained low but significant levels of IgG to recombinant gp63 and to soluble antigen, although there was no difference in the levels between constructs (specific IgG levels after one oral dose of GID101, GID105, and GID106 were 0.187 ± 0.005, 0.182 ± 0.006, and 0.207 ± 0.007, respectively, while mice administered PBS or BRD509 had background levels of 0.107 ± 0.005 and 0.103 ± 0.007 [mean units of optical density

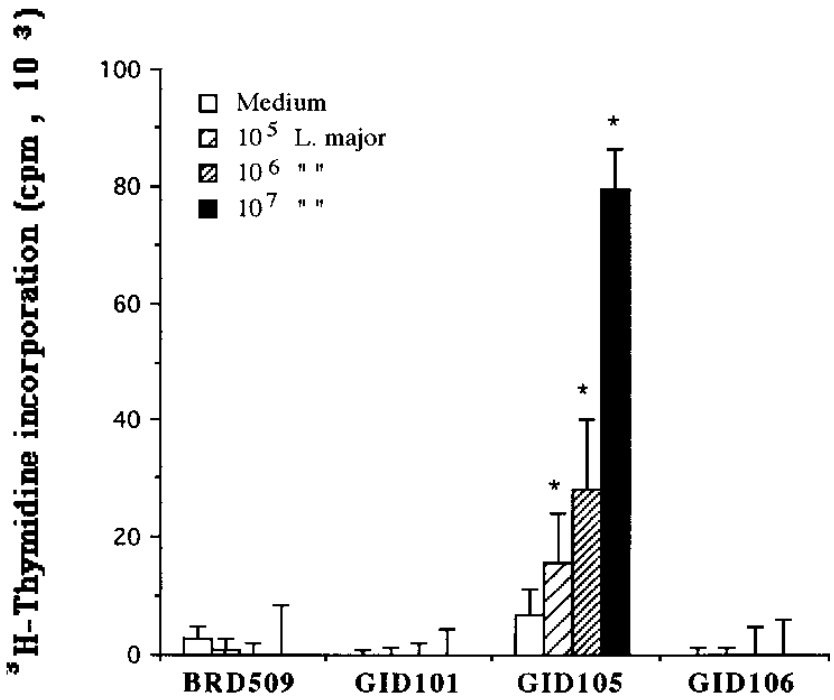

FIG. 5. Proliferative responses of spleen and lymph node cells from mice administered one oral dose of BRD509, GID101, GID105, or GID106 (10^{10} organisms/mouse). Cells were cultured for 4 days with live *L. major* in vitro. Means and standard deviations for triplicate cultures of pooled cells from three mice after subtraction of the background value in a culture with cells from mice inoculated with PBS alone are shown. The background value due to parasite proliferation was 67,670 cpm. The results are representative of two individual experiments. *, $P < 0.05$ compared to BRD509 values.

at 630 nm ± one standard deviation; 1/40 dilution; three mice per group; data are representative of three experiments). None of the mice developed any detectable DTH after injection in the footpad with live promastigotes (data not shown).

Antigen-specific immune response in vitro. BALB/c mice were given a single oral administration of GID105, GID106, GID101, BRD509, or PBS. Spleens and MLN were removed 3 to 4 weeks later and cultured in vitro with live *L. major* pro-

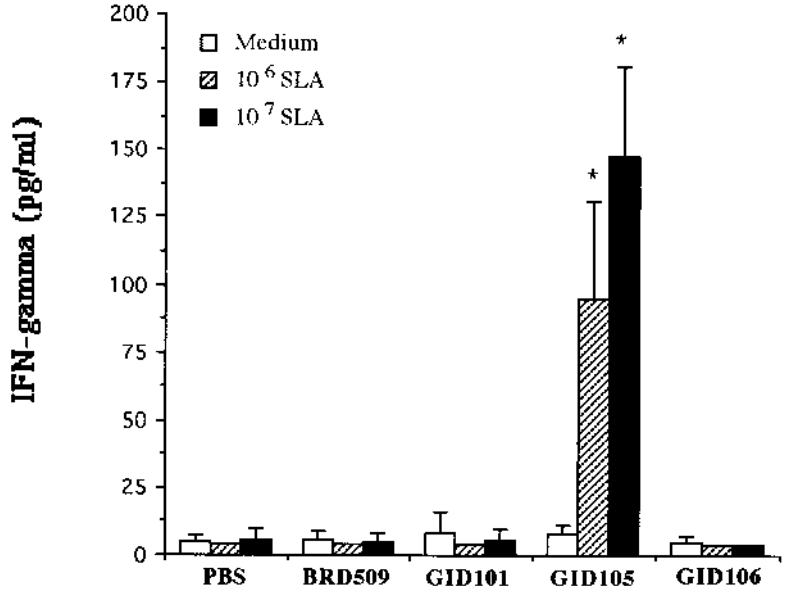

FIG. 6. IFN-γ production in vitro from cells stimulated with soluble leishmanial antigen (SLA). Mice were vaccinated orally with one dose of BRD509, GID101, GID105, or GID106 (5×10^9 organisms/mouse) or PBS. Spleen and lymph node cells were harvested 3 weeks later and cultured with graded doses of SLA. Supernatants were collected at 24, 48, and 72 h after incubation, and IL-2, IL-4, and IFN-γ levels were determined by ELISA. IFN-γ levels at 48 h are shown. Similar results were obtained at 72 h. Little or no cytokine was detected at 24 h. IL-2 and IL-4 were not detected at any time during the cultures (12 pg/ml). Data are means ± standard deviations for triplicate cultures of cells pooled from three mice and are representative of two individual experiments. *, $P < 0.05$ compared to BRD509 values.

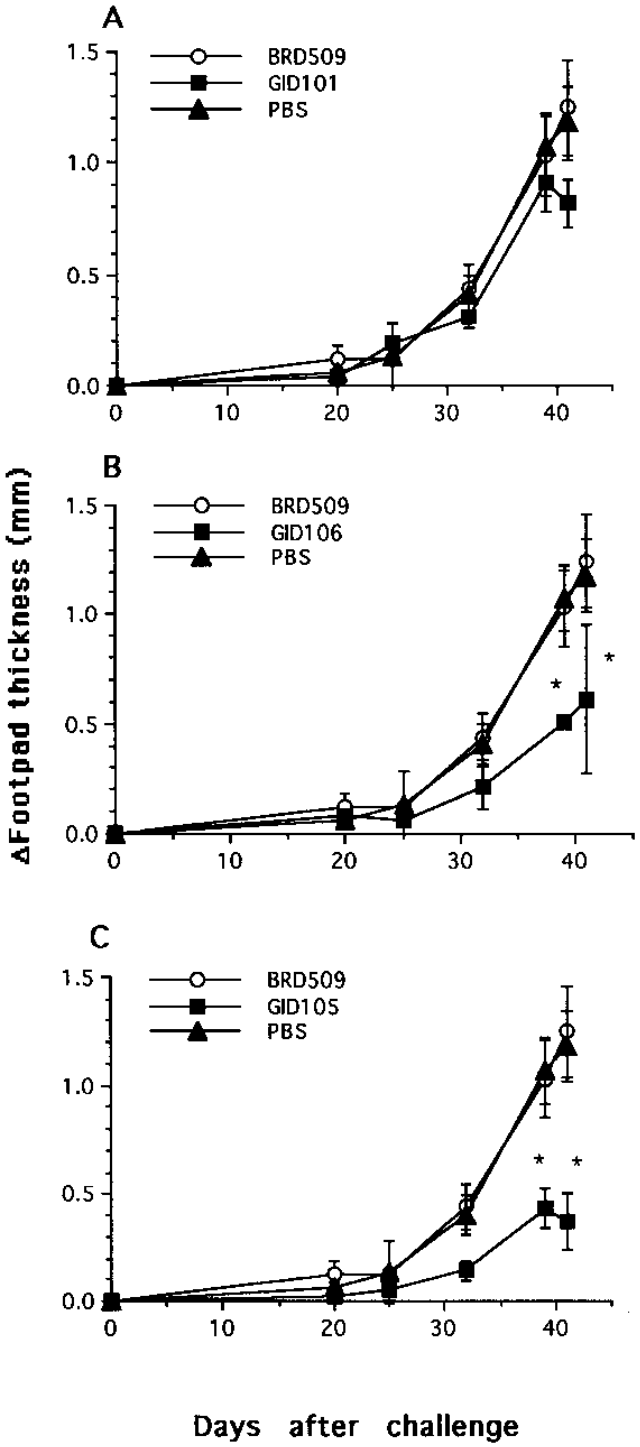

FIG. 7. Comparison of the protective effects of the three vaccine constructs. Mice were orally administered a single dose of BRD509, GID101, GID105, or GID106 (5×10^9 organisms/mouse) or PBS alone. They were challenged 3 weeks later in the footpad with 10^6 *L. major* promastigotes. Lesion development was measured at regular intervals as the increase in footpad thickness (subtracting the thickness of uninfected footpad from that of the infected footpad). Data are means ± standard errors of the mean ($n = 6$) and are representative of two experiments. *, $P < 0.05$ compared to BRD509 values. Experiments were terminated by day 42, when control mice (admistered BRD509) developed necrotic lesions in the footpad, as required by the current United Kingdom guidelines for animal experimentation.

mastigotes. Only cells from mice immunized with GID105 showed detectable dose-dependent proliferative responses (Fig. 5). Cells from mice administered GID105, cultured in the presence of soluble *L. major* antigens, also produced IFN-γ in a dose-dependent manner, whereas mice administered GID106, GID101, BRD509, or PBS did not (Fig. 6). IL-2 and IL-4 were not detected in any of the cultures.

Protection against *L. major* infection. BALB/c mice were vaccinated orally with 5×10^9 cells of either GID105, GID106, GID101, or BRD509. Control mice were administered an equal volume of PBS orally. Three weeks after administration, mice were challenged in the footpad with *L. major*, and lesion development was monitored. Mice vaccinated with one dose of GID101 did not show any reduction in lesion development (Fig. 7). In contrast, GID105-vaccinated mice had a considerable reduction in lesion development after a single oral dose. Mice given GID106 also displayed a significant reduction in lesion development after one dose, although this reduction was not as great as that observed with GID105. The lesion size in this system is directly correlated to the parasite loads in the lesion and the draining lymph nodes (data not shown and reference 39).

Protection against *L. donovani* infection. BALB/c mice were administered two oral doses of each construct (5×10^9 organisms in PBS) or were given PBS alone. Three weeks after the second oral dose, mice were challenged intravenously with 10^7 *L. donovani* amastigotes. At 2 and 4 weeks after infection, liver imprints were made from infected mice and slides were counted for numbers of amastigotes. Treatment of mice with GID105 conferred considerable protection against *L. donovani* infection at 2 and 4 weeks after infection, whereas GID106 or GID101 did not significantly alter the infection at these time points (Fig. 8).

DISCUSSION

The present study is aimed at improving the oral vaccine delivery system by using a live attenuated *Salmonella* carrier and comparing the relative efficacies of two inducible promoters. We have directly compared the efficacies of two in vivo-inducible promoters with that of a constitutive promoter, in terms of plasmid stability and immunogenicity, in order to gain an understanding of the critical parameters involved in the induction of immune responses with *Salmonella* strains. Data presented here show that the *nirB* promoter had significantly superior plasmid stability which correlated with increased immunogenicity to the heterologous antigen gp63.

The *nirB* promoter has been used to direct the stable expression of fragment C of tetanus toxoid (5). It is regulated by both nitrate levels and changes in oxygen tension in the local microenvironment. The promoter used here has been reconstructed to delete the nitrate-responsive regions and hence responds only to changes in oxygen tension. The regulation is effective, since no gp63 was expressed in aerobic growth but gp63 was readily detectable under reduced oxygen tension in vitro (Fig. 2).

The *osmC* promoter has been cloned and sequenced, but its regulatory elements remain unclear. This promoter responds mainly to elevated osmotic pressure, but the regulation is not tight, because it also responds to several other stimuli (10). Tartera and Metcalf (36) reported that the optimal osmolarity for *Salmonella* adherence and invasion of intestinal cells was at high NaCl concentrations (0.3 M). Since the osmolarity in the distal ileum, in which the bacteria initiate infection, is high, GID106 may express gp63 optimally before reaching lymphoid organs, accounting for suboptimal antigen-specific activation.

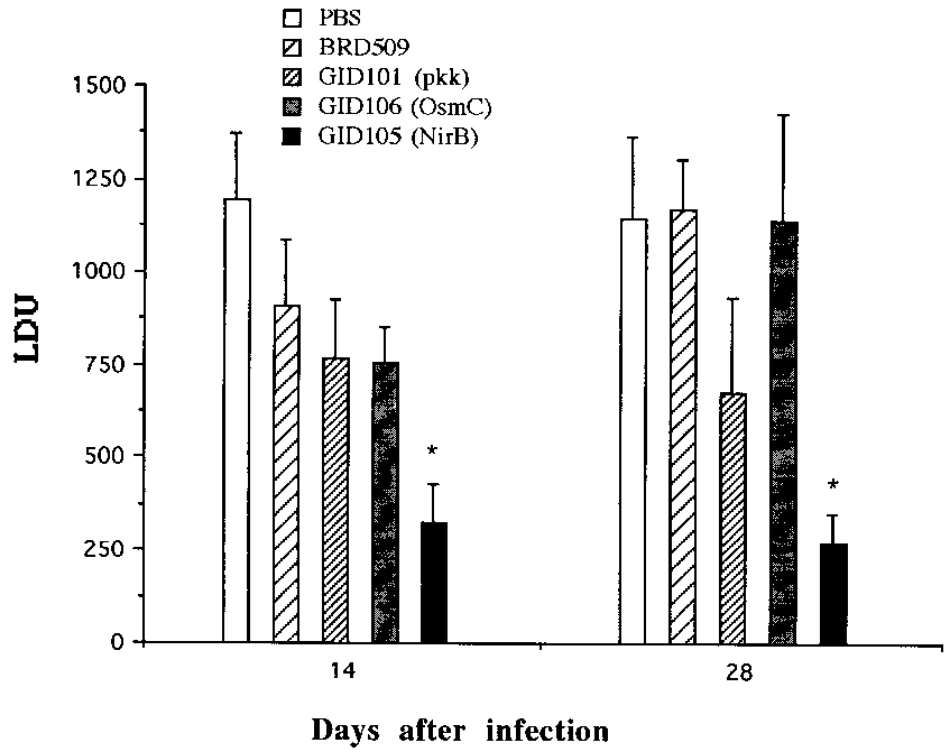

FIG. 8. Protection against *L. donovani* infection. BALB/c mice were administered two oral doses (2 weeks apart) of BRD509, GID101, GID105, or GID106 (10^{10} organisms/mouse) or PBS. Mice were challenged intravenously 3 weeks after the second dose with 5×10^7 *L. donovani* amastigotes per mouse. Mice were sacrificed on days 14 and 28 postinfection, and parasite numbers counted in the liver imprints were expressed as *L. donovani* units (LDU). Data show means and standard errors of the mean for individual mice per group. *, $P < 0.05$ compared to PBS controls.

The greater immunogenicity of GID105 is likely to be due to its superior stability as shown in vitro and in vivo. No plasmid was lost from GID105 after culture without ampicillin selection pressure for 98 generations in vitro. Under similar culture conditions, extensive plasmid loss was observed for GID101 and GID106. In vivo, GID105 plasmid stability was also found throughout the period of bacterial colonization.

However, increased plasmid stability was not the only factor noted after GID105 administration, as this construct also demonstrated different tissue tropism and increased bacterial loads compared to both GID101 and GID106. Colonization of the spleen and liver was undetectable after GID106 administration, and while colonies were readily found in the liver after GID101 vaccination, none of these bacteria contained the plasmid. Thus, delivery of gp63 by GID101 and GID106 was essentially confined to mucosal sites and may not have reached systemic antigen-presenting cells in sufficient quantities. An effect on tissue tropism was not observed in the TetC system and may be a specific effect due to the gp63 protein or a result of the use of higher inoculum doses in the TetC system. GID105 also colonized all organs at higher levels than GID101 or GID106, perhaps providing sufficient antigen to stimulate immune responses with a single dose. The different tissue tropism and bacterial load after GID105 administration may result from a relatively late induction of gp63 expression, thus allowing efficient bacterial colonization before the constraints of foreign protein expression occurred.

Significant cellular immune responses were detected only in mice immunized with a single dose of GID105. Splenic T cells from mice immunized with GID105 proliferated strongly against live *L. major* and secreted IFN-γ but no IL-4 when stimulated with leishmanial antigens in vitro. These responses were not detected in mice vaccinated with a single dose of GID101 or GID106. This is consistent with the general finding that protective immunity in the leishmanial system is mediated predominately by Th1 cells (11, 24, 31). However, DTH, a manifestation of Th1 cells, was not detected in any of the mice. This could reflect the relative insensitivity of the DTH assay. Despite the relative instability of GID106 and our inability to detect a Th1-like immune response induced by this construct, mice vaccinated with GID106 did develop a modest but significantly increased resistance against *L. major* infection. This suggests that other factors may have contributed to the resistance induced by GID106.

These data represent a significant advance in the development of a single-dose vaccine against leishmaniasis and also indicate that success in generating cross-species protection may depend on the expression system used. It should be noted that the challenge doses used here for both *L. major* and *L. donovani* are relatively high for BALB/c mice. Although gp63 may not be the most immunogenic of leishmanial antigens, it serves as an effective model antigen to demonstrate the potential efficacies of the different promoters used in the present study.

ACKNOWLEDGMENTS

This work received financial support from the United Nations Development Programme/World Bank/World Health Organisation Special Programme for Research Training in Tropical Diseases, The Medical Research Council of the United Kingdom, and the Wellcome Trust.

We thank J. Alexander, C. Carter, and D. Williams for technical assistance.

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Editor: S. H. E. Kaufmann